Mitochondrial Toxicity of Tenofovir Dipivoxil Fumaratein HK-2 Cells

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Abstract. Nucleoside analogs have been shown to have mitochondrial toxicity. In vitro tests can evaluate the mechanisms and severity of the toxicity. Tenofovir dipivoxil fumarate (TDF) is a novel drug for type B hepatitis. In this study, HK-2 cells were used to evaluate the effect of TDF on mitochondrial toxicity in the kidney. HK-2 cells were treated for 9 days in seven treatment groups. This study measured the inhibitory rate of cell proliferation, lactic acid levels, activities of mitochondrial respiratory chain complexes I–III, and mitochondrial DNA content as well as mitochondrial ultrastructure. Results exhibited that rates of cell proliferation, activities of complexes I, III and mitochondrial DNA content were reduced, but lactic acid levels increased in the 125 μM TDF group. Mitochondrial ultrastructure was damaged in the 125 μM TDF and 62.5 μM TDF groups. Thus, the 125 μM TDF group exhibited noticeable mitochondrial toxicity, whereas 62.5 μM TDF presented weak mitochondrial toxicity, and 31.25 μM TDF exhibited no obvious mitochondrial toxicity.

Introduction

Tenofovir dipivoxil fumarate (TDF) is a nucleotide reverse transcriptase inhibitor and a specific anti-hepatitis B virus drug candidate. Nucleoside analogs have been extensively used in the clinic to treat AIDS, type B hepatitis and herpes. Moreover, these types of compounds have an affinity for host cell DNA polymerase, which produces side effects in normal cells [4, 8, 32]. Numerous studies have confirmed that adverse reactions included myopathy (induced by azidothymidine), neuropathy (induced by d4t, ddi, and ddc), hepatic steatosis and lacticemia (induced by ddi, d4t, and azidothymidine), and peripheral lipoatrophy (induced by d4t), lactic acidosis, and pancreatitis [7, 8, 10, 16, 18, 24]. The above mentioned adverse reactions are caused by abnormal mitochondrial structure and function, including abnormalities of mitochondria-transcribed enzymes and the number of mitochondrial gene fragments [7, 21, 32], which can induce changes in mitochondrial structure, respiratory chain complex activity and mitochondrial DNA contents induced by azidothymidine[1, 12, 20, 26], ddc [11], ddi [6, 23], and d4t [23].

However, tenofovir disoproxil fumarate and tenofovir have been shown nephrotoxicity. A recent study suggest proximal tubular toxicity as a common pathogenic mechanism [33]. The clinical feature include acute kidney injury (toxic acute tubular necrosis), tubulopathies (Fanconi's syndrome, nephrogenic diabetes insipidus) and chronic kidney disease (chronic interstitial nephritis) [9, 15, 25, 28, 33]. Interestingly, mitochondria are key organelles in apoptotic cell death [27]. Early human studies and experimental evidence suggested that tenofovir itself was not associated with mitochondrial toxicity within the kidney [2, 5]. However, recent animal data demonstrate that tenofovir causes mitochondrial DNA depletion and mitochondrial toxicity[17, 19]. Therefore, the present study use HK-2 cells to research renal mitochondrial toxicity and severity of TDF treatment compared to azidothymidine, adefovir dipivoxil, and lamivudine, which were used as positive controls.

Materials and Methods

Drugs and Reagents

This study used TDF (Chia Tai Tianqing Co., Ltd., Jiangsu Province, China) molecular weight: 516.22, molecular formula: C21H35N5O8P, lot No. 20070618, content: 98.9%; positive control drugs: azidothymidine (Sigma, USA), molecular weight: 267.25, molecular formula: C10H13N5O4, batch No. 107K1578; adefovir dipivoxil (Sigma), molecular weight: 501.48, molecular formula: C20H32N5O8P, batch No. BCBC0377; and lamivudine (Sigma), molecular weight: 229.26, molecular formula: C8H11N3O3S, batch No. 069K47023. Solvent: dimethyl sulfoxide (Sigma), batch No. 064K0067. Dulbecco's Modified Eagle Media: Nutrient Mixture F-12(DMEM/F-12 medium, contain 2.5mm L-glutaminate and 15ml HEPES) and fetal bovine serum was purchased from Thermo-Fisher Biochemical Product Co., Ltd. (Beijing, China); trypsin (0.25% Trypsin + EDTA) was purchased from Hyclone Laboratories, Inc.. The reagents used to perform this study are as follows: animal tissue mitochondrial extraction kit; animal tissue mitochondrial dissolution kit; animal mitochondrial protein content detection kit; and animal mitochondrial respiratory chain complexes I–III activity quantitative detection kit (Genmed, Shanghai, China); axyprep genomic DNA mini kit (Axygen Biotechnology, Hangzhou, China); PCR kit (master mix), type B mini DNA fragment quick extraction kit, SYBR® Premix Ex Taq™ II (Perfect Real Time) quantitative PCR kit (takara Biotechnology (Dalian) Co., Ltd., Dalian, China).

Cell Line

Human HK-2 cell line (P10) was purchased from Xiehe Cell Resource Center, Beijing, China.

Test Equipment

The instrumentation to perform this study included the following: transmission electron microscope (H-600IV; Hitachi, Japan); Beckman high-speed refrigerated centrifuge; Thermo refrigerated centrifuge; Bio-Rad nucleic acid protein concentration detection apparatus; Fisher Scientific thermostatic water bath; Bio-Rad electrophoresis bath; Bio-Rad GEL EQ gel imaging system; and Bio-Rad icycler fluorescent real-time quantitative PCR system.

Grouping and Dosage

This study contained seven treatment groups. The TDF dose was established in accordance with an IC_{50} in HK-2 cells receiving TDF exposure for 9 days in our preliminary experiment. The IC_{50} of TDF was the highest concentration. The high-, moderate- and low-concentrations of TDF were 125μM, 62.5μM and 31.25μM, which were 197-, 99-, and 49-fold, respectively, the maximum serum concentration (0.63μM) in beagle dog orally taking a chronic dose of TDF at 3mg/kg. The clinical dose of TDF 100mg/person/day is approximately 3mg/kg for dogs according to body surface area. The final serum concentration of azidothymidine was 50μM, which was 7-fold the clinical maximum concentration (7μM) according to clinical drug concentration (100mg or 200mg/person/day) in patients with hepatitis B and AIDS [22, 31]. The final concentration of adefovir dipivoxil was 15μM, which was 223-fold the clinical maximum serum concentration (67nm) according to a clinical drug concentration (100mg or 150mg/person/day) in patients with hepatitis B and AIDS [3]. The final concentration of lamivudine was 30μM, which was 4-fold the clinical maximum serum concentration (8.3nm) according to clinical serum drug concentration (100mg/d/person) in patients with hepatitis B and AIDS [3]. The solvent control group used 0.1% dimethyl sulfoxide.

TDF, azidothymidine, adefovir dipivoxil, and lamivudine stock solutions were dissolved in medium containing 2.5% dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was between 0.0025% and 0.00025% in the drug treatment groups. The final concentration of dimethyl sulfoxide in the solvent control group was less than 0.1%.

Cell Culture and Treatment

HK-2 cells were cultured in DMEM/F12 medium. Different drug concentrations were added. There were three replicate flasks for each concentration. Cells were plated on 25-cm2 cell culture flasks and grown in a 5% CO2 incubator at 37℃ for 24h, the drugs were replaced by common medium. Subsequently, the medium changed every 3 days. Cells were maintained under these conditions for 9 days, respectively.

Inhibitory Effect on HK-2 Cell Proliferation

Following a 9 days drug treatment, Cells were removed by trypsinization and quantified by Trypan blue stain using a hemocytometer. The inhibition ratio formula is inhibition ratio=1- cell number in the treatment groups/cell number in the solvent control group ×100%. The solvent control group served as controls.

Effects on Lactic Acid Levels in HK-2 Cells

Following a 9-day drug treatment, the supernatant was collected, and cells were trypsinized. The number of living/dead cells was quantified using the Trypan blue exclusion method. The results were converted to lactic acid content per 106 cells to compare the levels of lactic acid under various concentrations.

Effects of TDF, Adefovir Dipivoxil, Azidothymidine and Lamivudine on Mitochondrial Structure

Following a 9-day drug treatment, 2-4 ml cells were centrifuged in a 1.5-ml centrifuge tube at 1500-2000 rpm (500 × g) for 10-15 min. The supernatant was discarded. The cells were fixed in approximately 0.5% glutaral (1:6 diluted in PBS), placed at 4℃ for 15-30 min, and centrifuged at 10,000-13,000 rpm (20,000 × g) for 10-15 min. After removal of the supernatant, the cells were fixed in 3% glutaral. The specimens were

prefixed in 3% glutaral, postfixed in 1% osmium tetroxide, dehydrated in acetone, and embedded in Epon 812. Semithin sections were subjected to optical localization. Ultrathin sections were stained with uranyl acetate and lead citrate. A transmission electron microscope (H-600IV; Hitachi) was used for observation.

Effects on Mitochondrial Respiratory Chain Enzyme Activity in HK-2 Cells

Following a 9-day drug treatment, cells were centrifuged at 1000 rpm (150 × g) for 10 min. After removal of the supernatant, mitochondria were extracted to detect total protein. An animal tissue mitochondrial extraction kit was used to measure mitochondrial respiratory chain complex activity according to the manufacturer's instructions.

Effects on Mitochondrial DNA Content in HK-2 Cells

Following a 9-day drug treatment, cells were centrifuged at 1000 rpm (150 × g) for 10 min. After removal of the supernatant, cells were quantified as abovementioned. Total DNA was extracted from 1×10^7 cells. Mitochondrial DNA and nuclear DNA contents were quantified using quantitative real-time PCR. The ratio of mitochondrial DNA to nuclear DNA in the solvent control group was considered the standard to assess for the effects of various drugs on mitochondrial DNA content.

Mitochondrial DNA content was measured using a quantitative real-time PCR kit according to the manufacturer's instructions. Cyt-b gene (forward primer, CATGATACCAATACGCAAATC; reverse primer, CGTGTGAGAGTGGGGCTGC) was used as a marker for mtDNA, while Actin gene (forward, CATCATGTTTGAGACCTTCAACACCC, reverse, CGTAGCTCTTCTCCAGGGAGG) was used as a marker for nuclear DNA. Primers were designed in accordance with the sequences of human mitochondrial Cyt-b gene and nuclear actin gene and synthesized by takara Biotechnology (Dalian) Co., Ltd. The PCR reaction was performed with the following conditions: one denaturation cycle at 94℃ for 5 min, followed by 40 cycles at 94℃ for 30 seconds, 56℃ for 30 seconds, 72℃ for 30 seconds, and end with an extension cycle at 72℃ for 10 min. The 25 μl reaction volume included 12.5 μl 2 × SYBR® Premix Ex Taq™, 1 μl of each primer (10 μM), 2.0 μl DNA template and 8.5 μl dh_2o. According to the nucleic acid concentration, retrieved Cyt-b and actin gene fragments were diluted to 12 gradient concentrations (10^{-1}-10^{-12}) using deionized water. Using the abovementioned reaction system, quantitative real-time PCR was performed, and a standard curve was constructed. DNA copy number was calculated according to the standard curve formula. The results were represented using the mitochondrial Cyt b gene to nuclear actin gene ratio.

Statistical Analysis

The data were expressed as the mean ± SD. Tukey-Kramer multiple comparison tests were used under normal distribution. The difference was analyzed using one-way analysis of variance. Kruskal-Wallis non-parametric test was utilized to measure the intergroup difference under normal distribution. All data were analyzed using NCSS 2000 software (NCSS, Kaysville, UT). A value of $P<0.05$ was considered statistically significant. Histopathological results were evaluated using qualitative indexes, such as swelling, disappearance, or reduction of mitochondrial crista.

Results

Inhibitory Effect on Cell Proliferation

The inhibitory test results following 9 days of TDF, adefovir dipivoxil and azidothymidine treatment in HK-2 cells are shown in Fig. 1. Cell numbers in the 125μM TDF, 62.5μM TDF, 31.25μM TDF, azidothymidine, adefovir dipivoxil and lamivudine groups decreased by 70.15±3.94% (P<0.01), 35.82±6.5%, 9.95±0.86%, 51.74±11% (P<0.05), 65.17±1.72% (P<0.01), and 39.3±4.56%, respectively, compared with the solvent control group.

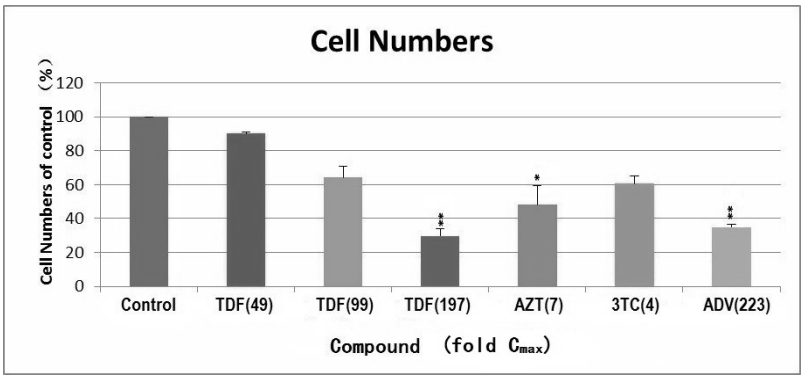

Figure 1 Effects of various drugs on cell numbers following a 9-day drug treatment in HK-2 cells. Cell cultures were exposed to 49-, 99- or 197-fold the maximum concentration (C_{max}) levels (Beagle dog) of tenofovir dipivoxil fumarate (TDF), and 7-, 223- or 4-fold the C_{max} levels of azidothymidine (AZT), adefovir dipivoxil (ADV) and lamivudine (3TC). Cell numbers were determined after 9 days. Values represent the percentage compared with 0.1% dimethyl sulfoxide (DMSO) vehicle-only control cultures and represent the means ± SD. *P<0.05; **P<0.01 (Dunnett's multiple-comparison test)

Effects on Lactic Acid Levels

Results of lactic acid contents in the supernatant following a 9-day drug treatment in HK-2 cells are shown in Fig. 2. Lactic acid levels in the125 μM TDF, 62.5μM TDF, 31.25μM TDF, azidothymidine, adefovir dipivoxil and lamivudine groups increased by 192.74±6.38% (P<0.01), 147.26±0.36%, 112.32±7.19%, 205.79±15% (P<0.01), 268.1±7.29% (P<0.01), and 193.47±16.85% (P<0.01), respectively, compared with the solvent control group.

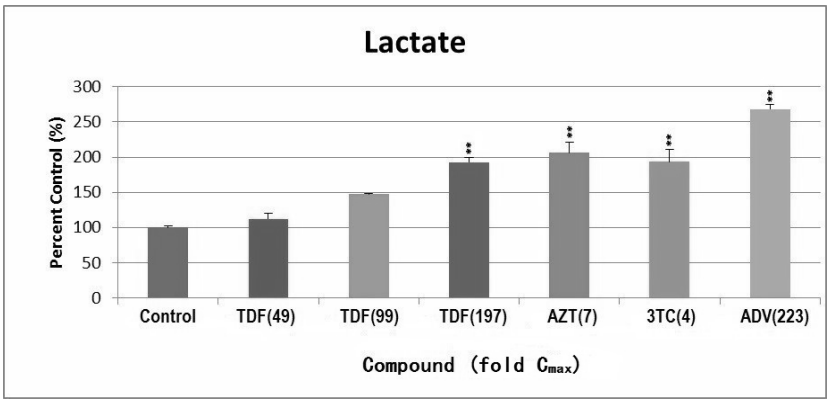

Figure 2 Effects of various drugs on extracellular lactic acid levels following a 9-day drug treatment in HK-2 cells. Cell cultures were exposed to 49-, 99- or 197-fold the Cmax levels (Beagle dog) of tenofovir dipivoxil fumarate (TDF), and 7-, 223- or 4-fold the Cmax levels of azidothymidine (AZT), adefovir dipivoxil (ADV) and lamivudine (3TC), respectively. Lactate levels were determined after 9 days and normalized according to cell numbers. Values represent the percentage compared with 0.1% dimethyl sulfoxide vehicle-only control cultures and represent the mean ± SD. *P<0.05; **P<0.01 (Dunnett's multiple-comparison test)

Transmission Electron Microscopy of Mitochondrial Ultrastructural

The results of transmission electron microscopy following 9 days of treatment of 0.1% dimethyl sulfoxide, TDF, adefovir dipivoxil, azidothymidine, and lamivudine in HK-2 cells are presented in Fig. 3. Abundant regular intact mitochondria and regular mitochondrial cristae were visible in the solvent control group. Intact mitochondria with a normal morphology were observed in the 31.25μM TDF group. Slightly swollen mitochondria and matrix dilution (low electron density) were observed, irregular cristae became short, and their number declined in the 62.5μM TDF and lamivudine groups. Moderately swollen mitochondria and decreased crista density were detected in the 125μM TDF group. Obvious swollen mitochondria, many electron-lucent areas in the matrix, dotted matrix with a vacuolar shape, crista disappearance, and mitochondrial membrane rupture were detected in the adefovir dipivoxil and azidothymidine groups.

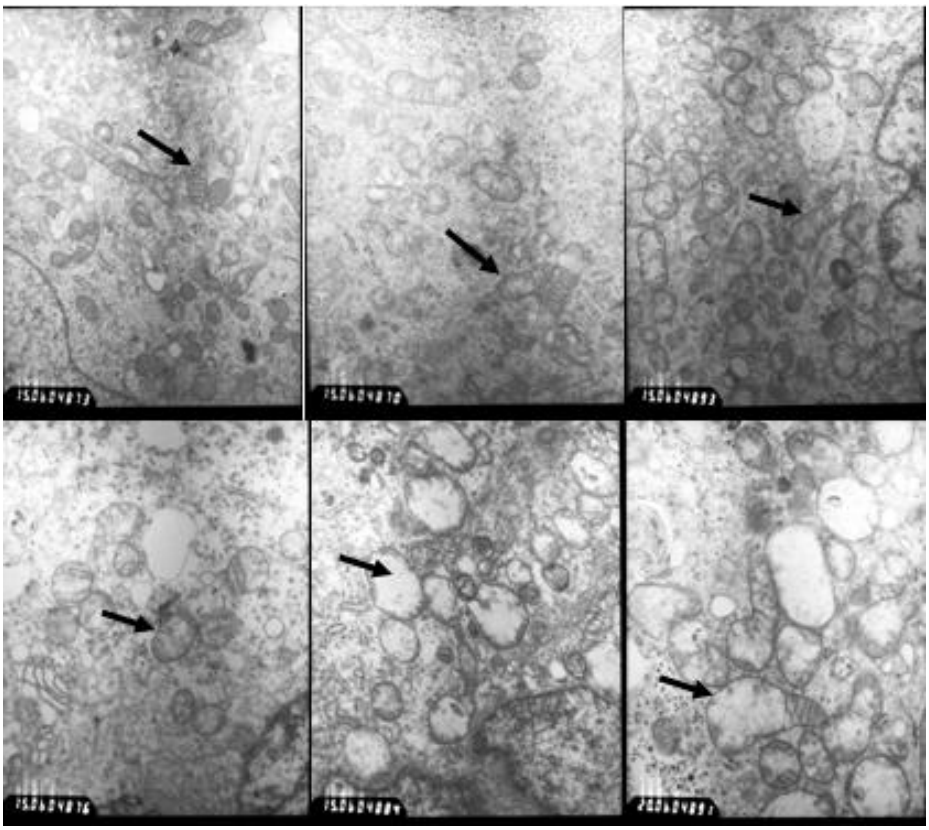

Figure 3 Effects of azidothymidine, adefovir dipivoxil, lamivudine and tenofovir dipivoxil fumarate (TDF) on mitochondrial structure under a transmission electron microscope (15000×). A, B: 0.1% dimethyl sulfoxide and TDF 31.25μM: abundant regular intact mitochondria and regular mitochondrial cristae with the absence of breakage or mitochondrial swelling; C: TDF 62.5μM and lamivudine 30μM: slightly swollen mitochondria and matrix dilution (low electron density), short irregular cristae with decreased number; D: moderately swollen mitochondria and decreased crista density; E, F: adefovir dipivoxil 15μM, azidothymidine 50μM: obvious swollen mitochondria, many electron-lucent areas in the matrix, dotted matrix with a vacuolar shape, crista disappearance, and mitochondrial membrane rupture.

Effects on Mitochondrial Respiratory Chain Enzyme Activity

The effects of TDF, adefovir dipivoxil, azidothymidine and lamivudine treatment on mitochondrial respiratory chain enzyme activity in HK-2 cells are presented in Table 1.

Table 1 Effects of tenofovir dipivoxil fumarate (TDF), adefovir dipivoxil, azidothymidine and lamivudine on the activities of mitochondrial respiratory chain enzyme activity in HK-2 cells (n = 3)

Group	Dose (μM)	Enzymatic activity (nmol/min/mg)		
		COX I	COX II	COX III
Solvent control	0.1% dimethyl sulfoxide	90.73±8.59	43.47±3.67	16.51±3.12
TDF	31.25	82.45±3.69	38.70±8.65	14.59±0.64
	62.5	57.06±4.91	35.88±9.71	12.66±2.61
	125	38.44±3.9*	25.57±11.94	5.79±0.33**
Azidothymidine	50	36.3±3.69*	26±6.06	5.16±0.44**
Lamivudine	30	26.95±5.52*	33.09±5.46	12.17±1.39
Adefovir dipivoxil	15	12.18±5.07**	16.66±2.86**	3.92±0.88**

Note: *P<0.05; **P<0.01 (Dunnett's multiple-comparison test)

Compared with the solvent control group, the activities of mitochondrial respiratory

chain complexes I was significantly lower in the 125μM TDF and azidothymidine groups ($P<0.05$). The activity of complex III was significantly decreased ($P<0.01$). The activity of complex II had a tendency to decrease, but there was no significant difference. The activities of mitochondrial respiratory chain complexes I was significantly reduced in the lamivudine group ($P<0.05$). The activities of complexes I–III in the adefovir dipivoxil group were significantly decreased ($P<0.01$). Both 31.25μM and 62.5μM TDF group decreased the activities of mitochondrial respiratory chain enzyme, but no significant difference was detectable.

Effects on Mitochondrial DNA Content

Quantitative real-time PCR results following 9 days of TDF, azidothymidine, adefovir dipivoxil and lamivudine treatment are listed in Table 2. Compared with the solvent control group, relative mitochondrial DNA content (cytochrome b/actin ratios) was significantly reduced in the 125μM TDF, azidothymidine, and adefovir dipivoxil groups ($P<0.01$). The decrease was also significant in the lamivudine group ($P<0.05$). The 31.25μM and 62.5μM TDF groups exhibited a decreased tendency, but there was no significant difference.

Table 2 Effects of tenofovir dipivoxil fumarate (TDF), adefovir dipivoxil, azidothymidine and lamivudine on mitochondrial DNA content in HK-2 cells (n = 3)

Group	Dose (μM)	Cyt b gene copies	Actin gene copies	Cyt b/Actin ratio (%)
Solvent control	0.1% dimethyl sulfoxide	$(1.22\pm0.17)\times10^{-7}$	$(1.29\pm0.05)\times10^{-5}$	0.954±0.169
TDF	31.25	$(5.43\pm1.81)\times10^{-8}$	$(1.07\pm0.03)\times10^{-5}$	0.506±0.166
	62.5	$(3.35\pm0.51)\times10^{-8}$	$(5.83\pm0.37)\times10^{-6}$	0.577±0.116
	125	$(3.69\pm0.88)\times10^{-9}$	$(3.41\pm0.33)\times10^{-6}$	0.109±0.030**
Adefovir dipivoxil	50	$(2.52\pm0.49)\times10^{-9}$	$(1.85\pm0.23)\times10^{-6}$	0.135±0.017**
Azidothymidine	30	$(3.82\pm0.54)\times10^{-9}$	$(2.50\pm0.24)\times10^{-6}$	0.153±0.015**
Lamivudine	15	$(2.39\pm0.17)\times10^{-8}$	$(5.47\pm1.12)\times10^{-6}$	0.447±0.077*

Note: *$P<0.05$; **$P<0.01$ (Dunnett's multiple-comparison test)

Discussion

The major findings of this experiment are mtDNA contents decreased, mitochondrial respiratory chain enzyme activity reduced, mitochondrial ultrastrucure changed and lactic acid levels increased. In this study, five aspects have developed to evaluate the renal mitochondrial toxicity and its severity of a novel drug -- tenofovir dipivoxil fumarate (TDF), and compared to the other three nucleoside drugs, use HK-2 cells *in vitro*. The results demonstrate that TDF in 125μM have significant mitochondrial toxicity.

The Cyt b/Actin ratio in TDF 125μM, adefovir dipivoxil, azidothymidine and lamivudine group have statistically significant decrease in this study, suggesting that the mtDNA contents in these groups have decreased. The activity of COX I in TDF 125μM and the other three positive control groups have statistically significant reduction; only in adefovir dipivoxil group the COX II activity reduced; the activity of COX III in TDF 125μM, adefovir dipivoxil and azidothymidine groups have

statistically significant reduction. Moderately swollen mitochondria and decreased crista density are observed in TDF 125μM of mitochondrial ultrastructural. The results are consistent with recent reports that tenofovir causes mitochondrial DNA depletion and mitochondrial toxicity, which is structurely similar to TDF [17, 19]. One study found that renal proximal tubules from HIV+ transgenic mice exposed to tenofovir showed ultrastructural mitochondrial abnormalities (irregular shapes with sparse, fragmented cristae) and decreased mtDNA levels, which paralleled the ultrastructural mitochondrial abnormalities [17]. Another study reported that rats exposed to tenofovir, proximal tubular dilatation, abnormalities in mitochondrial ultrastructure, depleted mtDNA, and depressed respiratory chain enzyme expression (cytochrome c oxidase and nicotinamide adenyldinucleotide dehydrogenase) were observed, and suggest that tenofovir causes mitochondrial toxicity within renal proximal tubular cells [19]. Distinctive proximal tubular eosinophilic inclusions representing giant mitochondria was observed by light microscopy in clinical tenofovir disoproxil fumarate therapy [15]. Ultrastructural finding is enlarged mitochondria; it may contribute to the long-term treatment.

In this study, the result of mitochondrial ultrastructural is consistent with mitochondrial respiratory chain enzyme activities. The mitochondrial toxicity degree in that concentration is adefovir dipivoxil>azidothymidine>TDF (125μM)>lamivudine. Adefovir dipivoxil and azidothymidine groups observed obvious swollen mitochondria, many electron-lucent areas in the matrix, dotted matrix with a vacuolar shape, crista disappearance, and mitochondrial membrane rupture; by contrast, TDF 125μM group showed moderately swollen mitochondria and decreased crista density; TDF 62.5μM and lamivudine groups only showed slightly swollen mitochondria and matrix dilution (low electron density), short irregular cristae with decreased number. The activity of COX I - III all reduced significantly in adefovir dipivoxil group (P<0.01); both COX I and COX III decreased in azidothymidine and TDF 125μM groups; only COX I activity reduced in lamivudine group. These results prove that the date is reliable and credible. In the experiment, quantitative real-time PCR results revealed that compared with the solvent control group, mitochondrial DNA contents were significantly reduced in the 125μM TDF group (P<0.01). Mitochondrial DNA contents in the 31.25μM and 62.5μM TDF groups exhibited a decreased tendency, but there were no significant difference. The decrease in mitochondrial DNA contents in HK-2 cells associated with mitochondrial toxicity were induced by 9 days of TDF exposure. Simultaneously, compared with the solvent control group, mitochondrial DNA contents were significantly reduced in the azidothymidine and adefovir dipivoxil groups (P<0.01). The decrease was significant in the lamivudine group (P<0.05). *In vivo* studies have verified that azidothymidine causes obvious mitochondrial dysfunction in human and other primates [13, 29, 34]. An *in vivo* study has confirmed that adefovir dipivoxil-induced nephrotoxicity is possibly mediated by a reduction in mitochondrial DNA [30]. *In vitro* cultured HK-2 cells, lamivudine can diminish mitochondrial DNA contents, but its effect was less than the effects of adefovir dipivoxil [4]. The results of lactic acid showed significantly increased in the 125μM TDF, azidothymidine, adefovir dipivoxil, and lamivudine groups (P<0.01), but there was no significant difference in the inhibition ratio of cell proliferation in the lamivudine group, suggesting that inhibition ratio was high in the 125 μM TDF, azidothymidine and adefovir dipivoxil groups. Lactic acidosis is one of the most serious complications induced by prolonged therapy with nrtis. A study confirmed that tenofovir have fatal lactic acidosis [14]. Azidothymidine and lamivudine has been shown lactic acidosis [4, 8].

This study has some shortcomings, such as short exposure time (15 days would be better), no dynamic observation, and the absence of effects on mitochondrial DNA polymerase.

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